Identification and Characterization of Chemoattractants for Epidermal Cells

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We have detected and partially characterized factors that promote the directed migration of mouse epidermal cells in a modified Boyden chamber assay. Smooth muscle cells grown in culture were found to secrete a potent chemoattractant for epidermal cells. This activity was further characterized and compared to the chemotactic activities found in wound fluid and conditioned medium from 3T3 L1 cells and with interleukin 1. The migration of epidermal cells during wound healing in vivo might be regulated by such factors. *J Invest Dermatol 90:122–126, 1988*

Epidermal cells make remarkable migrations during the healing of skin wounds. This may be an intrinsic property of the cells: epidermal cells present on a fragment of skin in culture will move to cover the entire surface of the explant in a process known as epiboly [1]. Such migrating cells are different from the stationary epidermal cells; they do not produce basement membrane components while migrating, but instead secrete other types of collagenous substrates [1]. Epidermal cells from the margins of the wound are not the only source of cells active in reepithelialization; epithelial cells present in defined portions of hair follicles and sweat glands also participate. The role of these latter cells may be particularly extensive when significant proportions of the superficial epidermis are destroyed [2]. It is generally thought that the loss of contact inhibition governs the migratory activity of epidermal cells [3], although most cells are able to respond to chemoattractants produced by other cells or released during clotting reactions, particularly in wounds [4,5]. Here we have adapted the Boyden chamber assay for studying the migration of epidermal cells and used it to screen for factors stimulating the directed migration of interfollicular and follicular mouse epidermal cells. Although both types of cells do not respond to a number of known chemoattractants (such as fibronectin, laminin and PDGF), we found that factors secreted by smooth muscle cells and interleukin 1 are potent chemoattractants for mouse epidermal cells.

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Abbreviations:

CM: conditioned medium
EC: epidermal cells
FCS: fetal calf serum
IL_1: interleukin 1
PDGF: platelet-derived growth factor
SMC: smooth muscle cells
SMCCM: smooth muscle cell conditioned medium
LOCA: low calcium

MATERIALS AND METHODS

Culture media prepared by the NIH Media Unit were supplemented with 0.1% of gentamicin sulfate, 2 mM glutamine, and 10% fetal calf serum from Gibco, Grand Island, New York. Trypsin, trypsin inhibitor, cycloheximide, heparin, and elastase were purchased from Sigma, St Louis, Missouri. The PDGF was obtained from Collaborative Research, Lexington, Massachusetts. Anti-$IL_1\alpha$ and $IL_1\beta$ antibodies were from Genzyme, Boston, Massachusetts, the BSA from Reheis Chemical Company Phoenix, Arizona, and Diffquick staining solutions were from Harleco, American Scientific Products, Columbia, Maryland. Nucleopore filters with a pore size of 8 μm were from Neuroprobe Corporation, Pleasanton, California; Dispase was from Bohreinger Manheim, Indianapolis, Indiana; collagenase type 1 from Worthington, Freehold, New Jersey; and DNAse type 1 from NBCO Biochemicals, Cleveland, Ohio. The 70-μm pore-sized gauzes were obtained through Martin Industries, Baltimore, Maryland. Unstimulated human blood monocytes supernatant fluids were provided by Y. Martinet (NHLBI: NIH, Bethesda, Maryland). Factor XIII was purified from human blood by S. I. Chung (NIDR: NIH, Bethesda, Maryland) and was studied either with or without thrombin activation. Chromatography resins were from Pharmacia, PL Biochemicals, Piscataway, New Jersey, and filtration membranes were from Diaflo, Amicon Corporation, Lexington, Massachusetts. Molecular weights markers were from Biorad, Rockville Center, New York. Fibronectin and laminin were purified as described previously [6,7].

Cell Cultures Full-thickness skin was removed under sterile conditions from 3-day old mice and floated overnight at 4°C on a solution of 0.25% Dispase in Hanks balanced salt solution. The epidermis of each skin was then separated from the dermis with a pair of forceps and used as a source of interfollicular epidermal cells, using Dispase to obtain an epidermal cell suspension. To isolate mouse hair follicles and mouse dermal fibroblasts, the dermis was digested on a rotary shaker at 37°C with culture medium containing 2 mg/ml of collagenase type 1, 0.01% of elastase, 100 μg/ml of DNAse type 1, and 10% FCS. At the end of the digestion (20 min/20 dermis), the material was filtered through cheese cloth to remove debris and then centrifuged at 29 *g* for 4 min. The pellet was retained as a source of hair follicles. The supernatant fluid was used as a source of fibroblasts. The cells were collected at 2000 rpm, washed twice with culture medium, and cultured at 37°C and 5% CO_2 in DMEM containing 10% FCS. The 29 *g* pellet, containing

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hair follicles, was washed twice with culture medium and centrifugated twice at 29 *g* to remove contaminating single cells. The supernatant fluids were discarded and the last pellet strained through a nylon gauze with 70-μm sized pores. The larger hair follicles stayed on the upper surface of the gauze while the smaller single cells passed through the gauze. The hair follicles were then washed from the gauze with culture medium and collected by low speed centrifugation. They were plated on petri dishes (50 hair follicles/ml) and grown in low calcium medium [8]. The purified hair follicles gave rise to follicular epidermal cell cultures, which expanded from the external root sheath of the hair follicle. To further characterize the epidermal nature of these cells, their insoluble proteins were extracted by the procedure of Steinert et al [9] and compared to standard interfollicular epidermal cell keratins. Follicular and interfollicular epidermal cells were cultured as in [10] in Eagle's medium number 2 supplemented with 10% chelaxed fetal calf serum. A low calcium medium contained between 0.02 and 0.09 mM $CaCl_2$ and a high calcium medium contained between 1.2 and 1.4 mM $CaCl_2$. The two epidermal cell types were used at their first passage after 3 or 4 days of culture. Other cell types used in this study were grown in DMEM containing 10% FCS, unless specified. Smooth muscle cells were isolated from bovine aorta (11); 3T3, 3T3L1, 1507 fibroblasts were obtained through American type collection; TR1, a characterized endothelial cell line, was kindly provided by J. Mather (Genentech, California). PAM 212 were generously supplied by P. Hawley-Nelson (NCI, NIH, Bethesda, Maryland) and were cultivated in the same manner as the epidermal cells.

Chemotaxis Assay To evaluate cell migration, we used a modified Boyden chamber composed of three parts: the lower blind well in which a test chemotactic substance was placed, the upper well where the cells challenged to migrate are suspended in the upper assay medium (500,000 cells/ml), and a porous filter interposed between the two chambers. Assay media were as follows: in the upper well, a low calcium culture medium containing 2 mg/ml BSA, and in the lower well a low calcium medium containing 0.2 mg/ml BSA (instead of serum). After 4 h in an humidified atmosphere of 5% CO_2 at 37°C, the chambers were opened and the filters were then fixed and stained (Diffquick). The cells that had migrated were retained on the lower face of the filter while the cells that had not migrated were wiped away from the upper part of the filter with a rubber policeman. Cell migration was quantitated by optical density in a multiscan apparatus with a 600-nm filter as previously reported [12]. All assays were done in triplicate, and the data reported here are the mean of these values. In addition, both positive and negative controls were run in each assay. Negative controls measured random cell migration in the absence of an attractant in the lower chamber, and this value was subtracted from the values obtained with directed migration. The number of migrated cells was expressed as a percentage of the positive control.

Checkerboard Analysis To determine whether smooth muscle cell conditioned medium (SMCCM) had true chemotactic activity (directed migration) or only chemokinetic (random migration) activity for epidermal cells, different concentrations of SMCCM were placed above and under the filters, in order to form varying concentration gradients of attractant [13].

Preparation of Conditioned Media Conditioned media were prepared from the cells as follows: cells were grown in their respective culture medium to confluency, then washed two times with serum free-medium and further incubated 24 h with the same medium. The resulting conditioned medium was centrifuged for 10 min at 10,000 rpm to remove the cell debris. The supernatant fluid was lyophilized and reconstituted at 1/10 of its original volume. At confluency, the number of cells in the different cell cultures varied by less than 10%. In order to use the same positive control for all experiments, SMC were grown in 1-L roller bottles; 100 ml of conditioned medium was prepared as above and aliquoted in 600 μl samples which were frozen and kept at −70°C until used.

Preparation of Wound Fluid Male Sprague-Dawley rats were subcutaneously implanted with wound chambers [14]. Wound fluid was aspirated after 5 days with a syringe and assayed for chemotactic activity.

Collagen Coating of Nucleopore Filters The Nucleopore filters were coated with the different collagens as previously reported [11] and tested for their ability to sustain epidermal cell migration with either laminin or fibronectin added in the upper Boyden chamber. Collagen I was extracted from rat tail [15]. Collagen IV was purified from the EHS tumor [7]. Collagen V was obtained from human placental membranes [11].

Characterization of the Smooth Muscle Cell Conditioned Medium Chemotactic Activity The production of chemotactic activity by cultured SMC cells was monitored, after cell confluency, every day for 8 days. The amount of the SMC chemotactic activity was also measured in cells cultured with either 10 μg/ml of cycloheximide or 5 or 25 μg/ml heparin. The potency of the SMCCM chemotactic activity was evaluated after successive dilutions. Sensitivity of the SMCCM to proteolytic cleavage was studied by treating positive controls with trypsin, and then with soybean trypsin inhibitor either immediately or after 30 min. The stability of the SMCCM factor was tested by heating it at various temperatures and also by dialyzing it against solutions of different pH. Its size and homogeneity were assessed by molecular sieve chromatography on a Sephacryl S200 column equilibrated with phosphate buffered saline without calcium and magnesium at pH 7.4. The effluents from the column were collected and tested for specific chemotactic activity for mouse epidermal cells and mouse dermal fibroblasts. Fibro-

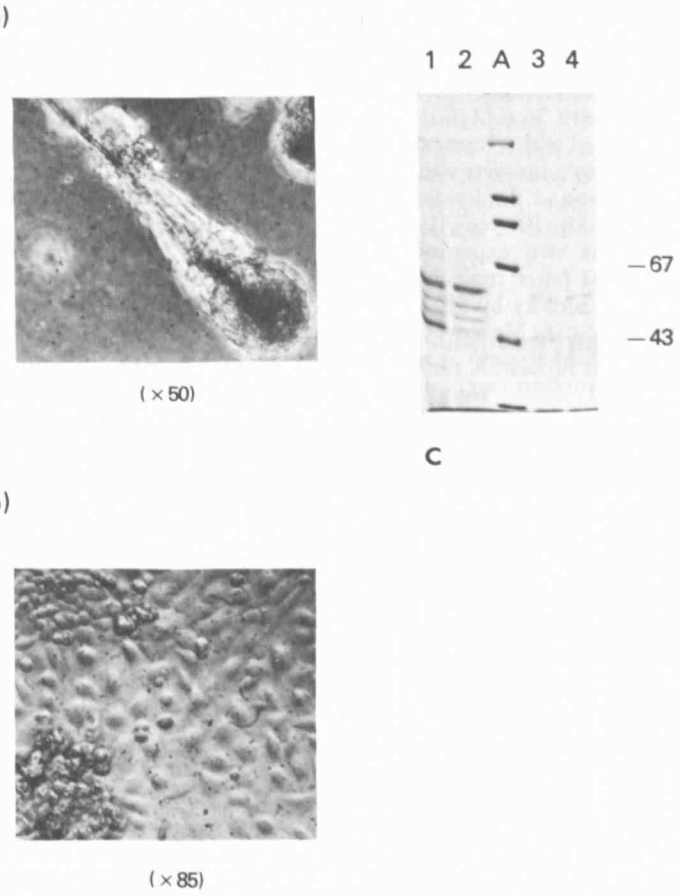

Figure 1. *(A)* phase contrast micrograph of hair follicles isolated from three day old mice (×50). *(B)* follicular epidermal cells in Loca medium after 3 days in culture (×85). *(C)* SDS-PAGE (8% polyacrylamide), *lane 1 and 2:* interfollicular epidermal cells; *lane 3 and 4:* follicular epidermal cells. Molecular weight *(lane A)* markers as indicated on the right side of the figure.

Table I. Migration of Epidermal Cells Toward Various Materials

Test Material	Epidermal Cells Chemotactic Activity[a]	
	Interfollicullar	Follicular
Conditioned Media Tested		
SMC	100	100
3T3L1	30	30
1507	10	10
Monocytes	0	0
TR1	0	0
B16 melanoma	0	0
NIH 3T3	0	0
Epidermal cells	0	0
Other Materials		
IL_1 (2μ/ml)	100	100
Wound fluid (undiluted day 5)	100	100
Wound fluid after affinity chromatography (unbound fractions)	100	100
Human serum (10%, 5%, 0.5%)	0	0
Laminin (1, 100 μg/ml)	0	0
Fibronectin (1, 50 μg/ml)	0	0
PDGF (1, 25 ng/ml)	0	0
Factor XIII activated or not by thrombin.	0	0

[a]In percent.

nectin was removed from the SMCCM by affinity chromatography using a gelatin Sepharose affinity column equilibrated with 0.15 M NaCl, 5 mM EDTA, 10 mM Tris-HCL (pH 7.4). A 5 ml positive control sample of SMCCM was loaded over the column. After binding was completed (UV monitoring), the column was eluted with 4 M urea. Aliquots of unbound and bound fractions were assayed for chemotactic activity. The unbound fraction was serially chromatographed on an heparin Sepharose affinity column equilibrated with 25 mM Tris-HCL (pH 7.4) containing 0.1 M NaCl, and eluted with a linear salt gradient (0-2 M NaCl). Unbound and bound fractions were also assayed for chemotactic activity. A sample of rat wound fluid was also processed identically. To study the relationships between IL_1 and the chemotactic activity contained in SMCCM two experiments were performed. Partially purified SMCCM (after molecular sieve chromatography) was tested for IL_1-like activity by SK Durum (NIH, Frederick, Maryland) as in Reference 16. Moreover, IL_1 antibodies ($IL_1\alpha$ or $IL_1\beta$) were added to SMCCM aliquots at various dilutions (1/10, 1/20, 1/30, 1/40) and the mixtures were incubated overnight at 4°C. Resulting chemotactic activities of SMCCM aliquots incubated in this fashion

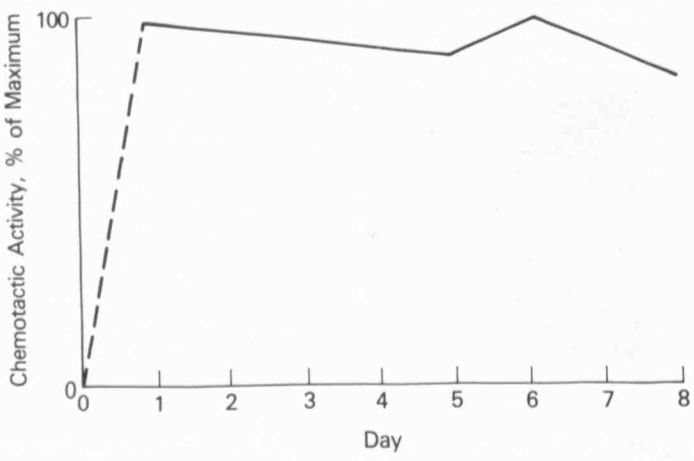

Figure 2. Secretion by cultured SMC of a chemotactic activity for interfollicular epidermal cells. SMC were grown in their culture medium to confluency. Every day for 8 days, one set of triplicate SMCCM was tested for chemotactic activity.

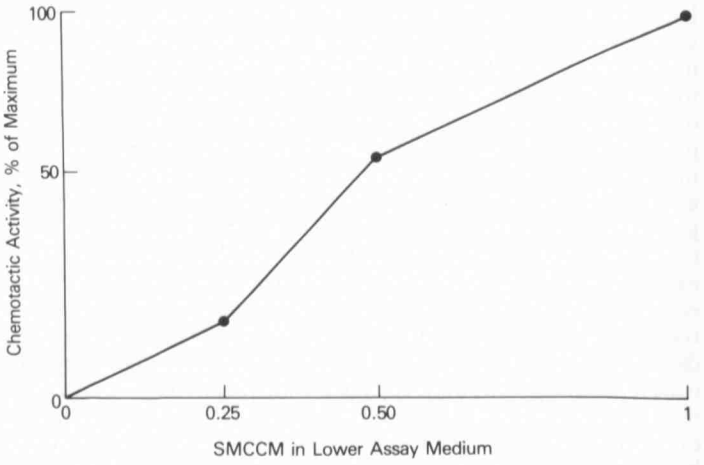

Figure 3. Dose-response curve of epidermal cell chemotaxis toward SMCCM. Positive controls in triplicate were serially diluted in the lower chamber assay medium and assayed for chemotactic activity toward interfollicular epidermal cells grown in Loca medium.

were next measured in the Boyden chamber assay. These activities were compared to the chemotactic activities of untreated SMCCM and activities of lower medium assay in which antibodies alone were added at the same dilutions and incubated at 4°C overnight.

RESULTS

Viable epithelial cells were readily obtained from cultured hair follicles isolated from the skin of three day old mice. These cells grown on plastic closely resembled typical epidermal cells and their pattern of keratin peptides was also very similar (Fig. 1). When tested for chemotactic activity, both cell types had a similar responsiveness. No response was noted with a variety of substances that are chemotactively active with fibroblastic cells, including fibronectin, laminin, PDGF, serum or conditioned medium from monocytes. Conditioned medium from cultured 3T3, B16 melanoma, or endothelial cells were also devoid of activity, but SMCCM, as well as media from 3T3 L1 and human fibroblasts, demonstrated activities (Table I). Of these sources, the SMCs secreted the most potent activity. This activity from the SMCCM was characterized further. The SMCs produced their chemoattractant in culture at a continuous rate for 8 days (Fig 2). Different dilutions of SMCCM produced a dose-related response in the migration of the epidermal cells (Fig 3). The activity of the SMCCM was assessed by a checkerboard assay (Fig 4). Cell migration was highest when the concentration of attractant was higher in the lower compartment as expected for a true chemoattractant. Some increase in cell migration was also observed with equal amounts of attractant on both sides of the filter,

SMCCM Dilution in Upper Well

SMCCM Dilution in Lower Well	1/4	2/4	3/4	4/4
1/4	10.5 ± 0.2	0	0	0
2/4	30 ± 2	10 ± 1.4	10 ± 0.7	0 ± 3.2
3/4	60.4 ± 2.3	30 ± 3	20.3 ± 0.3	10.1 ± 0.6
4/4	80 ± 1.7	50.2 ± 1.2	30 ± 0.7	20.5 ± 1.2

Figure 4. Checkerboard analysis of SMCCM chemotactic activity for epidermal cells: two assays were performed with triplicate filters and the results are their mean value.

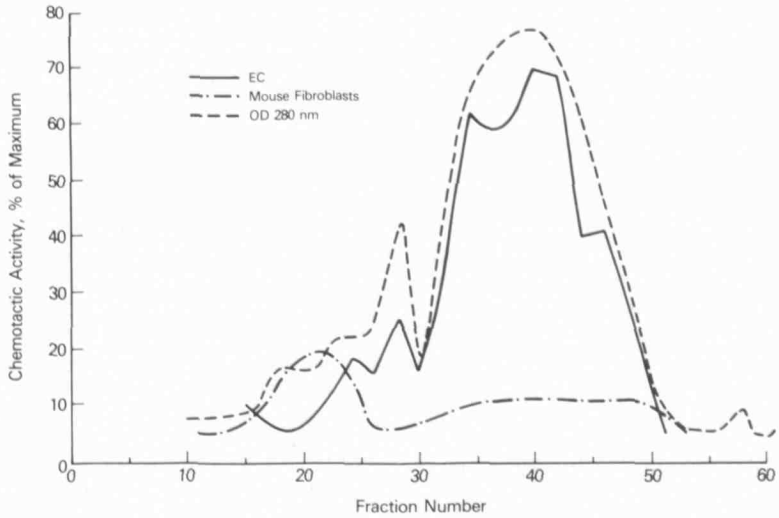

Figure 5. Chromatography of the SMCCM: 5 ml of SMCCM were concentrated by ultrafiltration on P10 amicon membrane and were chromatographed on a Sephacryl 200 column. Three-milliliter fractions were collected, dialyzed into the lower chamber assay medium before testing for chemotactic activity with mouse interfollicular epidermal cells and with mouse dermal fibroblasts. The column size was 1.6 × 37 cm; total column volume; 74 ml; void volume: 24 ml. Standardization: BSA: Kd: 0.254 (mol: 67,500); ovalbumin: Kd: 0.364 (mol wt: 43,000); chymotrypsinogen: Kd: 0.534 (mol wt: 25,000); ribonuclease: Kd: 0.640 (mol wt: 13,700). Peak chemoattractant activity for fibroblasts: Kd: 0.18. Peak chemoattractant activity for epidermal cells: Kd: 0.57 and Kd 0.78. The *curves* show the chemotactic activities for each cell population and the optic density at 280 nm. EC are mouse epidermal cells.

indicating that this factor also has some chemokinetic activity. SMCCM was applied to a molecular sieve column, and aliquots from the column effluent were assessed for chemotactic activity towards fibroblasts and epidermal cells. The chemotactic activity for epidermal cells emerged as a single rather broad peak of some 10 to 20,000 daltons. The major chemotactic activity for fibroblasts was present at 80 to 100,000 daltons (Fig 5). Several observations—molecular size, loss of activity when heated and absence in conditioned media from cells treated with either cycloheximide or heparin—suggested that the SMCCM chemotactic factor for epidermal cells was a polypeptide. On the other hand, it was not destroyed by trypsin and was not bound by gelatin or heparin as might be expected, for example, of fibronectin fragments (Table II). We also tested other factors for their ability to promote epidermal cell migration. In vivo, chemoattractant(s) for epidermal cells were also present in rat wound fluid (Table I) and were also unrelated to fibronectin (data not shown). We lastly found that interleukin 1 (IL_1) possessed a potent chemotactic activity, while factor XIII (whether activated or not by thrombin) did not. The SMCCM attractant for epidermal cells did not seem to be related to interleukine 1 since anti-IL_1 antibodies (anti-human $IL_1\alpha$ or $IL_1\beta$) failed to significantly inhibit the migration of epidermal cells toward SMCCM (SMCCM: 100% migration; SMCCM + IL_1 antibodies at a dilution of 1/10: 70% migration; $IL_1\alpha$ or $IL_1\beta$ antibodies alone: 10%). Moreover, no IL_1 biological activity was found when using SMCCM at a large range of dilutions in the mitogenic assay, which measures thymidine uptake in the mouse D10 lymphocyte target cell (normal uptake in the presence of IL_1: 30,000 cpm; in the presence of SMCCM: 3,000 cpm; in the absence of IL_1: 500 cpm). By varying the substrates on which epidermal cells migrated, we saw that most types of collagen were able to sustain epidermal cell migration (Fig 6). Fibronectin promoted epidermal cell migration on collagen type I while laminin promoted epidermal cell migration on collagen type IV. In contrast, fibronectin when added to the epidermal cell suspension diminished their migration on collagen type IV and V. Neither laminin or fibronectin were by themselves chemotactic for epidermal cells nor was migration seen when we used uncoated filters. Interfollicular epidermal cells and follicular

Table II. Characterization of SMCCM Chemotactic Activity After Varied Treatments of SMCCM or SMC Cultures

Treatments of SMCCM	Chemotactic Activity[a]
Lyophilization Heating	
37°C	90
56°C	27
100°C	25
Trypsin Digestion[b]	100
pH stability:	
2	100
4	100
6	100
8	100
Addition of anti-IL_1 α- or β-antibodies (1/10–1/40)	70
Gelatin Sepharose chromatography:	
unbound	100
bound	0
Heparin Sepharose chromatography:	
unbound	100
bound	0
Treatments of SMC Cultures with:	
cycloheximide (10 μg/ml)	0
heparin (5, 20 μg/ml)	0

[a] In percent.
[b] 100 μg/ml of trypsin, inhibited 30 min later by 2.5 mg/ml of soyabean trypsin inhibitor.

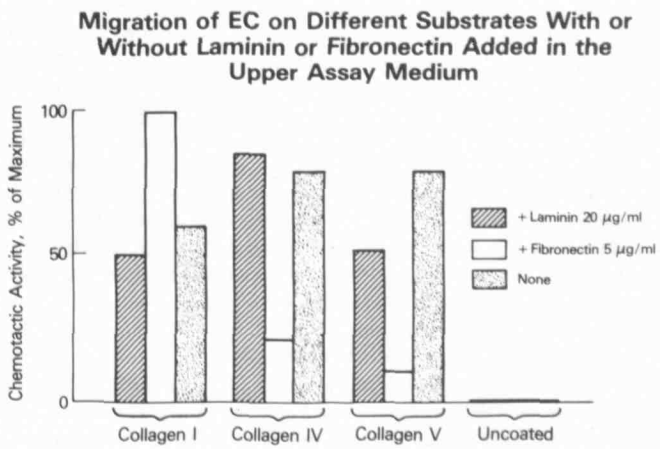

Figure 6. Interfollicular epidermal cell migration on different types of collagen, coated filters with or without laminin or fibronectin added to the different cell suspensions. Nucleopore filters were coated with different types of collagen as in (8) and tested for their abilities to sustain epidermal cell migration. Three assays were carried out. In the first assay, 5 μg of fibronectin was added to the cell suspension; in the second assay, it was replaced by 10 μg of laminin and in the last assay, no addition was made. The results are represented as means of triplicate filters and are compared with the last set of uncoated filters. The maximal migration obtained was taken as 100%.

epidermal cells demonstrated similar behavior when grown in low calcium medium or switched to high calcium medium. The number of migrating cells was reduced by 75% for both cell populations in the latter case (data not shown). PAM 212 cells, a mouse epidermal cell line, showed no chemotaxis towards any factor described here (data not shown).

DISCUSSION

It is likely that in an effort to close a wound, most epithelia repair in the same way: first by cellular migration and then by cellular proliferation. We report here that the epidermal cells migrate toward several attractants, including a very potent one secreted by SMC, which is unrelated to fibronectin or IL_1. Similar activities were present also in the fluid of experimental wounds in rats. Conceivably, any cell underlying the epidermis could have a role in epidermal cell migration by producing such attractants. Strong interactions between epidermis and dermis during embryogenesis are well documented [2]. Indeed, a potent epidermal cell chemotactic activity was produced by SMC, but not by other dermal cells. As SMC are present all the time in the dermal blood vessels, wounding might trigger their secretion of attractants. Myofibroblasts appearing specifically during wounding [17], and sharing both fibroblastic- and SMC-like characteristics, could also be a potential source of chemoattractants. Another candidate for an epidermal cell migration activity is factor XIII [18]. We did not find any activity for factor XIII in our assay. Our finding that IL_1 possesses chemotactic activity for epidermal cells is interesting because epidermal cells as well as macrophages secrete IL_1 [19]. However, we could not attribute the chemotactic activity found in SMCCM to an IL_1-like molecule. The in vitro observations that epidermal cells can attach and migrate on different types of collagen substrates correlate well with in vivo observations where the presence of a unique specialized substrate is not evident during the early stages of wound repair [20–23]. It has been suggested, however, that a continuous secretion of collagen is required for the continuation of epidermal cell migration [24]. Our results support such views because no epidermal cell migration was observed when the filters were uncoated and more than one substrate was able to sustain epidermal cell migration. The role of fibronectin during epidermal migration has been emphasized [25]. During our studies, we added fibronectin to the migrating cells and found it to be an enhancer of cell migration by allowing spreading of epidermal cells on collagen-coated filters. However, neither fibronectin nor laminin were by themselves chemoattractants for these cells. Both interfollicular and follicular epidermal cells were able to migrate in vitro as they do in vivo, but differentiated cells obtained after culture in high calcium did not migrate to the same extent as did basallike cells cultured in low calcium medium. The low calcium conditions promote the proliferation of cells with unorganized desmosomes that are more ready to migrate than differentiated cells cultured in high calcium medium. PAM 212, a transformed cell line, did not demonstrate comparable migrating capacity during our studies. This points out the necessity to use normal cells for confirmation of results obtained with cell lines. Such in vitro studies might in the future help to define factors able to improve reepidermization during wound healing in vivo.

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